CLIN. CHEM. 26/7, 854-860 (1980)

Total Serum Cholesterol by Isotope Dilution/Mass Spectrometry: A Candidate Definitive Method

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We describe a highly accurate and precise method for determination of total cholesterol in serum by isotope dilution/mass spectrometry. The method was developed for a Study Group of the Committee on Standards of the American Association for Clinical Chemistry, for use in establishing the accuracy of a candidate reference method for total cholesterol, and fulfills their criteria for a definitive method.

Cholesterol-d_7 is added to serum, with the weight ratio of cholesterol-d_7 to total serum cholesterol kept near to 1:1. The esters are hydrolyzed and the cholesterol is separated and converted into the trimethylsilyl ether derivative for measurement by combined gas chromatography/mass spectrometry. The intensity ratio of the molecular ions at m/z 465 and 458 is measured for each sample and for two calibration mixtures, according to a prescribed bracketing protocol. A weight ratio for the sample is obtained by linear interpolation of the ion-intensity ratios, and the total cholesterol is then calculated.

The method was applied four times over several weeks to each of five serum pools. Statistical analysis involving consideration of both replication error and variability between weeks gave a coefficient of variation for a single measurement of 0.36%. The absence of interferences in the method was demonstrated by measurements at several other masses.

As a participant in a Study Group organized under the Committee on Standards of the American Association for Clinical Chemistry (AACC), whose objective was to develop a reference method of known accuracy for total serum cholesterol, our laboratory was to provide a definitive method, i.e., a method with which the accuracy of the reference method could be established by intermethod comparison (*1, 2*). The Study Group, chaired by Dr. G. R. Cooper (Center for Disease Control Atlanta, GA 30333),[4] suggested as a desirable accuracy goal for the definitive method a coefficient of variation (CV) no greater than ±0.5% and a total uncertainty no greater than 1% for serum pools having total cholesterol concentrations ranging from 3.5 to 8.5 mmol/L (about 1.35 to 3.75 g/L).

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Identification of any commercial product does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the material or equipment is necessarily the best available for the purpose.

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Received Jan. 14, 1980; accepted Mar. 21, 1980.

Our experience with the accurate analysis of serum glucose (*3*), uric acid, and urea suggested that the required accuracy should be obtainable by isotope dilution/mass spectrometry (ID/MS), even though such high accuracy had not been demonstrated by Björkhem et al. (*4, 5*) or Sieckmann et al. (*6*) with their total cholesterol ID/MS methods, nor more recently by Gambert et al. (*7*) or Mee and Halpern (*8*). Some of those authors (*4, 6, 7*) considered their methods to be suitable for use as reference methods, but the AACC study group regarded ID/MS as a potential definitive method, and in its search for a reference method preferred to consider methodologies more familiar to clinical chemists (*9*).

The first use of ID/MS methodology as a definitive method for clinical chemistry was by Cali et al. (*10*), to evaluate the accuracy of an atomic absorption spectroscopy method as a reference method for total calcium (*11*). Moore and Machlan (*12*) described the details of the ID/MS method for calcium that involved thermal ionization mass spectrometry, a technique appropriate for ID/MS of inorganic elements. Thermal ionization ID/MS methods, though mostly not yet published as definitive methods, are now being applied to the determination of chloride (*13*), iron, lead, lithium, magnesium, and potassium (*14*). In particular, they are used in evaluating reference methods for these elements.

We describe here an ID/MS method for total cholesterol in serum: Isotopically labeled cholesterol is added in an amount about equal to the approximately known quantity of total cholesterol in the sample; cholesterol esters are hydrolyzed; and the mixture of labeled and unlabeled cholesterol is extracted and converted into trimethysilyl ethers. The ion-intensity ratio of the molecular ions at m/z 465 and 458 is determined by gas chromatography/mass spectrometry (GC/MS) according to a prescribed measurement protocol that includes the sequential measurement of calibration mixtures composed of trimethylsilyl ethers of labeled and unlabeled cholesterol whose ratios closely bracket the observed ratio for the sample. The total cholesterol in the serum is calculated from the quantity of serum, the quantity of labeled cholesterol added, and the weight ratio of labeled to unlabeled cholesterol found by the measurements. Evidence for the precision of the method is presented, and we offer our estimate of the limit of its possible bias. Although evidence is provided also to show that the measurements are interference-free, one or more other laboratories must test this method or develop other methods that are equal or better, if we are to judge the magnitude of its bias with more assurance.

Materials and Methods

Serum Samples

Vials of frozen human serum (−60 °C), selected without conscious bias from eight different pools, were provided by Dr. Gerald R. Cooper (Center for Disease Control) (*15*). At NBS, three of the pools were labeled Pools I–III and five pools were labeled Pools A–E, and these samples were stored at −20 °C. The relative density (specific gravity) of each serum pool was determined by weighing samples in a calibrated 0.5-mL Lang–Levy glass pipet on a semi-microbalance (*16*).

Cholesterol-d_0 [Cholest-5-en-3-ol(3β)].

We used Standard Reference Material (SRM) no. 911a, certified by the National Bureau of Standards to be 99.8 ±0.1% cholesterol, as the cholesterol standard. Approximately 10 mg (weighed to ±10 μg) was dissolved in about 7.9 g of absolute ethanol (solution weighed to ±0.01 mg) in a 10-mL volumetric flask, to serve as a stock solution for preparing calibration mixtures. Changes in concentration over the period during which the solution was used were minimized by stoppering the flask with a thimble-shaped polyethylene cap, through which the needle of a syringe could be inserted for withdrawing aliquots.

Cholesterol-d_7 [Cholest-5-en-25,26,26,26,27,27,27-d_7-3-ol(3β)]

This material was obtained from Applied Science Laboratories, Inc., State College, PA 16801. It was purified by sublimation at 144–148 °C and 8 Pa (~0.06 mmHg), and recrystallized from a mixture of chloroform and methanol at room temperature. A single crop of the recrystallized cholesterol-d_7 was collected and washed with methanol.

The mass spectrum of the purified cholesterol-d_7 showed an intensity ratio for m/z 393 to m/z 386, indicating the presence of less than 0.1% of cholesterol-d_0. GC/MS (after trimethylsilylation as described below) revealed 1 to 2% of a 24-carbon steroid impurity, probably chol-5-en-3-ol(3β) derived from the starting material for the cholesterol-d_7 synthesis. Stock standard solutions of cholesterol-d_7 in ethanol were prepared (as described above for cholesterol-d_0) for use in calibration mixtures and for additions to serum samples.

[4-^{14}C]Cholesterol and [4-^{14}C]Cholesteryl Oleate

These compounds, obtained from New England Nuclear, Boston, MA 02118, were tested for radioactive impurities by thin-layer chromatography followed by radioautography.

For radioautography we used 20 × 25 cm No-Screen Medical X-ray film, liquid X-ray film developer and replenisher, and rapid fixer, all from Eastman Kodak Co., Rochester, NY 14650.

The thin-layer chromatographic plates were 20 × 20 cm, precoated with a 250-μm layer of silica gel G, and were from Analtech, Inc., Newark, DE 19711.

Other Chemicals

Only ACS-reagent-grade chemicals were used. The chloroform and methanol used in recrystallizations were redistilled. The redistilled chloroform was stabilized by adding 7.5 mL of methanol per liter. We tested the methanol and absolute ethanol for the presence of substances that could interfere with the measurements by evaporating 0.25 L of each, adding the silylating reagent to the residue, and performing GC/MS analysis. "Aquasol-2" scintillant fluid, used in liquid scintillation counting, was from New England Nuclear.

Sampling of Solutions: Weighed-Aliquots

Aliquots of sera and ethanol solutions were measured by weighing in "conditioned" plastic syringes. The plungers of these 2.5-mL syringes with Luer-Lok® tips were covered with 0.17-mm (0.007-inch) Teflon® film before they were inserted into the syringe barrels, to minimize contamination (*12, 17*). Needles were 8- or 13-cm, 19-gauge stainless steel. Conditioning consisted of filling and draining a clean syringe with liquid sample three times. The quantity of an aliquot was determined by weighing the refilled syringe and weighing the emptied syringe after the aliquot was transferred into the receiver. The aliquot-weighing technique had been used previously for serum samples (*12*) but not for ethanol solutions. With solutions containing 10 mg of cholesterol and 2 μCi of [4-^{14}C]cholesterol in 10 mL of ethanol, we found by measuring the radioactivity of 10 separate weighed aliquots (about 1 mL each) that the CV for the average radioactivity per gram of aliquot was 0.16%. This value includes a 0.10% CV ascribable to the random counting error. With use of an unconditioned syringe, the radioactivity per gram of the first three of 10 aliquots gradually approached the highly precise value we found for the subsequent seven.

Table 1. Weight Ratios of Cholesterol-d_7 to Cholesterol-d_0 in Some Calibration Mixtures, Showing the Limited Range of Compositions Used for Bracketing Measurements

Calibration mixture no.	Weight ratio d_7/d_0
1	0.8297
2	0.8880
3	0.9088
4	0.9423
5	0.9961
6	1.0205
7	1.0629
8	1.0999

Calibration Mixtures

Weighed aliquots of the cholesterol-d_7 and -d_0 stock standard solutions were combined to prepare a series of solutions whose ratios by weight of cholesterol-d_7 to -d_0 were between 0.8 and 1.2. Table 1 lists the weight ratios of the calibration mixtures used in the within- and between-day precision study described below. These calibration mixtures were used in pairs in a bracketing pattern for the analysis of serum samples. As an illustration, Table 2 shows the pairs of calibration mixtures used with the eight serum aliquots of one serum pool that were analyzed as part of the precision study described below.

We tested the self-consistency of this series of calibration mixtures by extracting from all available data every occurrence of a calibration mixture being immediately bracketed by other calibration mixtures, neglecting intervening samples. We calculated a weight ratio for each bracketed calibration mixture, assuming that those used for the bracketing were accurate. This cross-comparison between calibration mixtures indicated that the measurement of the calibration mixtures was in control. No departure from linearity was observed.

Sample Preparation (Wet-Chemistry for ID/MS): Sampling of Serum and Hydrolysis of Cholesterol Esters

Two vials of frozen serum from each of Pools A–E or eight vials from Pools I–III, as needed to contain the required amount of cholesterol, were allowed to thaw and reach room

Table 2. Pairs and Order of Use of Calibration Mixtures Used for the First-Day Measurements to Bracket the Eight Aliquots (Two Aliquots in Each of Four Sets) for the Analysis of One Serum Pool by ID/MS[a]

	Calibration mixtures	
Set no.	Aliquot 1	Aliquot 2
1	8,7	2,4
2	2,1	4,6
3	3,4	5,7
4	4,3	4,5

[a] *The calibration mixtures are characterized in Table 1.*

temperature. After gently mixing and combining, aliquots were taken, each of sufficient volume to contain about 1 mg of total cholesterol: two aliquots of pools A–E and eight to 10 of pools I–III. Each aliquot was placed in a 45-mL standard tapered centrifuge tube that contained a weighed 1-mL portion of the cholesterol-d_7 stock solution. From this point on, the volumes of added solvents and reagents were not critical, because all quantitation is related to the *ratio* of cholesterol-d_0 to cholesterol-d_7. To each tube we added 0.6 mL of 8.9 mol/L aqueous potassium hydroxide and 4 mL of absolute ethanol, and kept each mixture at 37 °C for 3 h. Then we added 5 mL of water and 10 mL of hexane, vigorously mixed (vortex-type mixer) the combination for 1 min, and separated and evaporated the hexane layer. The solid residue, consisting mainly of cholesterol-d_7 and -d_0, was dissolved in 2 mL of methanol for storage and subsequent handling.

These conditions for hydrolyzing cholesterol esters, a modification of those used by Abell et al. (*18*), were adopted after the following tests were run: A solution of 1 mg of cholesterol oleate and 0.83 μCi of [4-^{14}C]cholesterol oleate in 30 mL of ethanol and 1.8 mL of aqueous potassium hydroxide (8.9 mol/L solution) was mixed and divided into 5-mL portions. The portions were kept at 37 °C for 0.5, 1, 2, 3, 4, or 24 h. Then each was extracted with hexane and the products obtained on concentration of the extract were separated by thin-layer chromatography on silica gel with benzene/ethyl acetate (9/1 by vol). Radioautography for three days and, in separate experiments, spraying the chromatographic plates with sulfuric acid and charring revealed no cholesterol oleate remaining after 1 h of hydrolysis and showed cholesterol to be the only product; no other new radioactive or nonradioactive products were detected. With mixtures of [4-^{14}C]cholesterol and cholesterol similarly treated, we found no evidence of cholesterol decomposition. In both experiments, less than 0.1% of a radioactive degradation product would have been detected by the radioautography test.

Derivatization of Cholesterol

A 50-μL aliquot of the methanol solution of the cholesterol that had been extracted from a serum sample or 100-μL of a calibration mixture was evaporated to dryness. The residues were treated with 50 μL or 100 μL of bis(trimethylsilyl)acetamide, respectively, at room temperature for at least 0.5 h before analysis by GC/MS. Samples and standards thus converted into the cholesterol trimethylsilyl ether derivative and stored were found to remain unchanged on storage at room temperature when tested over several months.

GC/MS Instrumentation

We used a Model CH 7A mass spectrometer (Varian MAT, Florham Park, NJ 07932) equipped with a combined chemical ionization/electron impact ion-source, a multi-ion selection device, and a Varian Model 2740 gas chromatograph, with modifications and additions as noted. The standard ion-detection system for the CH 7A was used, including the electron multiplier, preamplifier, and amplifier. The output of this amplifier is connected to three devices in parallel: a mass-peak display, the multi-ion selection device (the output of which was used only for qualitative data), and a multi-channel scaler for quantitative data.

The mass-peak display device permitted the selection and observation of a region of the ion beam about one atomic mass unit wide. From a low-frequency function generator, we applied a voltage of triangular wave form (55 Hz, with ±20 V maximum amplitude) to the CH 7A beam-deflection plates located in front of the exit slit, so that an ion-intensity signal was obtained by sweeping a small mass range. This signal was displayed on an oscilloscope, providing continuous visual monitoring of peak shapes and positions. The advantages for quantitative application of combining continuous display with selected ion monitoring have been previously noted for systems having other methods of display (*19, 20*).

The multi-ion selection device controlled the switching of the magnetic field, for monitoring at the masses of d_0- and d_7-cholesterol, and the timing, for acquisition of intensity data. The device also provided for acquisition of intensity data for each mass selected, and for the data to be displayed in the form of gas-chromatographic peak profiles.

Data for quantitation were collected in parallel by two channels of an eight-channel scaler-timer (built at NBS), which converts voltage to frequency and then counts, giving 100 counts per millivolt-second. Two masses were monitored alternately, and the separately accumulated counts were transferred to a Model 9830A Calculator (Calculator Products Division, Hewlett-Packard Co., Loveland, CO 80537) for data reduction.

GC/MS Procedure

We injected about 3 μL of the cholesterol trimethylsilyl ether reaction mixtures (representing about 1.5 μg of cholesterol-d_0) onto a 125 × 0.31-cm (o.d.) stainless-steel column packed with 1.5% OV-101 on Chromosorb G, 100/120 mesh (Applied Science Laboratories, Inc.). Helium flow was about 22 mL/min. The temperatures of the injection port, column, and interface to the mass spectrometer were 270, 250–265, and 250–260 °C, respectively. As the column aged, the column temperature was adjusted to maintain a retention time for the cholesterol trimethylsilyl ether of about 6 min. A subset of the samples was also analyzed by use of a GC column packed with 3% OV-17 on Gas-Chrom Q, 100/120 mesh (Applied Science Laboratories, Inc.), at 275 °C.

Measurements in the electron impact mode were made at an ion-source temperature of 250 °C and an ionization energy of 70 electron volts. For ammonia chemical ionization, the source temperature was 200 °C, with an indicated ion-source pressure of 5 mPa (4×10^{-5} mmHg).

The multi-ion selection device was set to monitor two selected ions alternately for one second each by switching the magnetic field. The chosen magnetic field switching rate (*a*) minimized the loss of intensity signal that occurs during switching and (*b*) permitted the acquisition of a minimum of 70 samplings for each mass, thus ensuring sufficient data for the desired precision (*21*). The peak-display device was operated simultaneously, with the width of the display set so that for each mass peak all of that peak and some of the adjacent baseline on each side were displayed. We collected intensity data just before elution of the gas-chromtographic peak began in order to establish a background correction. The data were then collected until the intensity for cholesterol-d_0 declined to 1% of the maximum. We separately summed the intensity data for the two masses, corrected for background, and calculated their ratio.

Measured intensity ratios were found to depend somewhat on whether data collection was terminated at an intensity of 10, 5, or 1% of the maximum intensity. This was expected, because some separation was effected by the chromatography, i.e., the cholesterol-d_7 peak maximum occurred a few seconds before the cholesterol-d_0 maximum. Because both the samples and calibration mixtures are affected identically, no dependence of the calculated weight-ratios was expected or observed if the intensity as a fraction of the maximum at the termination of data collection was kept constant for both samples and calibration mixtures.

All of the cholesterol trimethylsilyl ether reaction mixtures were analyzed by electron impact mass spectrometry by measuring the ratios of the molecular ions ($M^{+\cdot}$) at *m/z* 465 and 458. A subset of samples was also analyzed by measuring the ratio of (*a*) the $(M - TMS{\cdot}OC_3H_4)^+$ fragment ions at *m/z*

Table 3. Summary of Precision of Within-Day ID/MS Analyses of Three Serum Pools

Pool	Av concn, mmol/L	n [a]	CV, % (*single measurement*)
I	6.083	16	0.24
II	6.746	20	0.16
III	8.325	18	0.24

[a] Includes two valid measurements per aliquot analyzed.

336 and 329 in the electron impact mode, and (*b*) the $(M + NH_4 - TMSOH)^+$ ions at *m/z* 393 and 386, obtained by ammonia chemical ionization. (TMS represents the trimethylsilyl group.)

ID/MS Measurement Protocol and Calculations

1. Each calibration mixture or sample was measured twice in succession. The two observed intensity ratios were acceptable only if they agreed to within 1%; if not, a third measurement was performed, which had to agree with the second, and the three were averaged. Their average constituted a valid measurement. If a calibration mixture was used again during any given half-day, only a single additional ratio measurement was made at each use if the new value was within 1% of the previously measured value. The observed gas-chromatographic peak heights for all calibration mixtures and samples within any given half-day were required to be within a factor of two of each other.
2. Measurements were made in the following order: lower weight-ratio calibration mixture, sample, higher weight-ratio calibration mixture (or the reverse), so that each valid sample measurement was bracketed by valid measurements for two calibration mixtures.
3. On a second day the same sample and bracketing mixtures were remeasured in the reverse of the order initially used.

We calculated the weight-ratio of cholesterol-d_7 to -d_0 in the sample by linear interpolation between the bracketing mixtures, using equation 1, which is obtained from the three possible linear equations of the form RW = A + B(RI) by the elimination of the constants A and B:

$$RW_S = \frac{(RI_S - RI_L)\,(RW_H - RW_L)}{RI_H - RI_L} + RW_L \qquad (1)$$

where S refers to the sample, H and L refer to the bracketing calibration mixtures having the higher and lower weight ratios, respectively, RW is the known weight ratio of cholesterol-d_7 to cholesterol-d_0 in a calibration mixture or the weight ratio to be determined in a sample, and RI is the measured valid intensity ratio.

C, the molar concentration of cholesterol-d_0 in the sample, was then calculated as follows:

$$C = \frac{(M^*)\,(D_S)}{(M_S)\,(RW_S)}\left(\frac{P}{386.66}\right) \qquad (2)$$

where M^* is the weight of cholesterol-d_7 added to the sample, M_S is the weight of the serum sample, D_S is the specific gravity of the serum, RW_S is calculated from equation 1, P is the known fractional purity of the cholesterol-d_0 Standard Reference Material, and 386.66 is the relative molecular mass of cholesterol.

Results

ID/MS Method: Within-Day Precision

We analyzed three serum pools (Pools I–III) as follows. Between eight and 10 separately weighed aliquots from each pool were combined with weighed aliquots of a cholesterol-d_7 stock standard solution, and the mixtures were taken through all the wet-chemistry steps within one day. (For that reason these results are referred to as "within-day" precision data.) Ion-intensity ratios were measured at *m/z* 465 and 458, according to the ID/MS measurement protocol. Table 3 summarizes the total cholesterol concentrations found.

ID/MS Method: Within- and Between-Day Precision

Two weighed aliquots were taken from each of five different serum pools (Pools A–E) for analysis as a set. Four such sets were analyzed; the sampling and wet chemistry for each set was begun at about one-week intervals. A fresh standard stock solution of cholesterol-d_7 was used as the internal standard for each set. Ion-intensity ratios were measured at *m/z* 465 and 458, according to the measurement protocol. The results are given in Table 4. The individual values listed are the valid measurements for each day.

Statistical Analysis of the Within- and Between-Day Data

We made an analysis of variance of the results in Table 4 for each group of four measurements corresponding to a given set for a given pool. The analysis showed that neither days nor aliquots had any effect. In view of these findings, the four measurements were considered as four replicates. For the purpose of a statistical analysis of the data, Table 4 can be considered to consist of five separate groups, one for each pool, and a one-way analysis of variance was carried out separately for each pool.

The results of the statistical analysis are shown in Table 5. The column headed "Average" represents the grand mean for each pool. The standard deviations for "Replication" and "Sets" were obtained as follows. The four standard deviations of replication calculated from the four sets of each pool were pooled and the resulting root-mean-square values were linearly regressed on the corresponding pool averages. The fitted

Table 4. ID/MS Analytical Results: Within- and Between-Day Precision Study of Serum Pools A–E

Set	Day	A Aliquot 1	A Aliquot 2	B Aliquot 1	B Aliquot 2	C Aliquot 1	C Aliquot 2	D Aliquot 1	D Aliquot 2	E Aliquot 1	E Aliquot 2
		Cholesterol concn, mmol/L									
1	1	3.4251	3.4393	4.7133	4.7280	6.1536	6.1298	7.4345	7.4498	8.7806	8.7860
	2	3.4432	3.4331	4.7133	4.7115	6.1391	6.1360	7.4764	7.4441	8.7646	8.7814
2	1	3.4297	3.4303	4.7285	4.7337	6.1641	6.1391	7.4645	7.4524	8.8061	8.7816
	2	3.4197	3.4336	4.7265	4.7177	6.1641	6.1448	7.4622	7.4547	8.7992	8.7873
3	1	3.4122	3.4132	4.6960	4.6986	6.1350	6.1030	7.4361	7.4366	8.7733	8.7553
	2	3.4173	3.4143	4.6976	4.7038	6.1414	6.1301	7.4271	7.4149	8.7610	8.7336
4	1	3.4416	3.4328	4.7383	4.7275	6.1819	6.1708	7.4900	7.4560	8.8469	8.8177
	2	3.4483	3.4434	4.7383	4.7319	6.1752	6.1827	7.5078	7.4820	8.8188	8.8167

Table 5. Statistical Summary for the Within- and Between-Day Precision Study

Pool	Cholesterol concn, mmol/L: Average	Standard deviation (mmol/L): Replic. [b]	Sets	Reprod. [c]	CV, %: Replic. [b]	Sets	Reprod. [c]	Standard error of average [a]: mmol/L	CV, %
A	3.4298	.005910	.011316	.012765	.17	.33	.37	.0058	.17
B	4.7190	.008132	.014597	.016710	.17	.31	.35	.0076	.16
C	6.1494	.010597	.020877	.023413	.17	.34	.38	.0108	.18
D	7.4556	.012848	.021850	.025348	.17	.29	.34	.0114	.15
E	8.7881	.015144	.028046	.031873	.17	.32	.36	.0145	.16
Av.	—	—	—	—	.17	.32	.36	—	.16

[a] Standard error of average = $\sqrt{(s^2_{Replic.}/16) + (s^2_{Sets}/4)}$.
[b] Values are smoothed by means of a linear regression of the observed standard deviations on the averages.
[c] Reproducibility standard deviation = $\sqrt{s^2_{Replic.} + s^2_{Sets}}$.

Table 6. ID/MS Results Obtained by Measurements with Other Selected Ions

Note	m/z pair	Day	Pools: A	B	C	D	E
			Cholesterol concn, mmol/L				
1	465/458	1	3.4297	4.7285	6.1391	7.4645	8.7816
		2	3.4197	4.7265	6.1448	7.4622	8.7873
2	465/458	1	3.4204	4.7164	6.1262	7.4622	8.8033
		2	3.4259	4.7131	6.1486	7.4575	8.7610
3	393/386	1	3.4253	4.6932	6.1461	7.4425	8.7145
		2	3.4112	4.6950	6.1138	7.4082	8.7145
4	336/329	1	3.4153	4.7089	6.1404	7.4379	8.7695
		2	3.4181	4.7190	6.1456	7.4487	8.7648

Notes: (1) Data from Table 4.
(2) Electron impact/MS measured on $M^{+\cdot}$ as in (1), but seven months later, and chromatographed on an OV-17 column.
(3) Ammonia chemical ionization/MS measured on the $(M + NH_4^+ - TMSOH)$ ion, (TMS = trimethylsilyl).
(4) Electron impact/MS as in (1), measured on the $(M - TMS{\cdot}OC_3H_4)^+$ ion, (TMS = trimethylsilyl).

values are recorded in the column headed "Standard Deviation for Replication." The standard deviation for "Sets" was obtained by the following formula:

$$s_{Sets} = \sqrt{s^2_{\bar{x}} - \frac{\hat{s}^2_{Replic.}}{4}} \qquad (3)$$

where $s_{\bar{x}}$ is the standard deviation between the four averages for each pool, and $\hat{s}_{Replic.}$ is the value in the previous column.

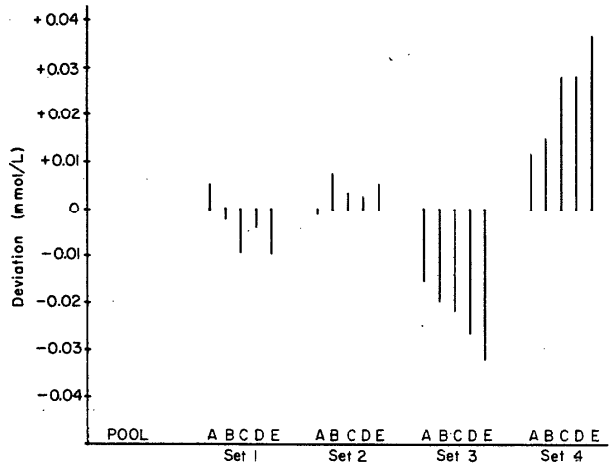

Fig. 1. Plot of the deviations of the averages for total cholesterol concentration for each pool in a set from the grand concentration averages of the pools
The pools are arranged in ascending order of total cholesterol concentration from left to right in each set

Figure 1 illustrates the deviations of each cell average from the overall pool average for that material.

ID/MS Method: Results by Measuring Other Ions

Five samples from the within- and between-day series were analyzed by measuring ion-intensity ratios of other ion pairs in the mass spectra of the cholesterol-d_7 and -d_0 tremethylsilyl ethers. The samples consisted of one each from the five pairs of aliquots in set 2 of Pools A–E that had been analyzed with use of their m/z 465 to 458 ion-intensity ratios. Table 6 lists these ion-pairs, the measurement conditions used, and the results obtained.

Discussion

In isotope-dilution analysis, the unknown quantity of an analyte is determined by completely mixing the sample with a known quantity of an isotopically labeled form of that analyte and then measuring the ratio of the two forms by an isotope-discriminating technique. Usually a portion of the mixture of the two analyte forms is more or less completely separated from other substances, to help assure that the measurements are entirely specific. The results of the analysis should be highly accurate if the two forms of the analyte mix completely before any separation is done, if the ratio of the forms remains unaltered during the processing of the sample (as is expected because the difference in the forms is due only to the presence of isotopic atoms in the labeled molecules), and if the measurements are suitably calibrated and interference-free. This rationale assumes appropriate accuracy in all the other aspects of the procedure.

Mass spectrometry is especially appropriate for performing isotope-dilution analysis, because with it separate signals for the labeled and unlabeled forms of the analyte can be mea-

sured concurrently and their ratio thus obtained. In the case of organic analytes, the coupling of a gas chromatograph to the mass spectrometer provides a convenient means for effecting a final purification step and for introducing the mixture of analyte forms into the mass spectrometer. Although the combination provides the basis for highly specific analysis, there are many sources of analytical error that must be avoided if accuracy is to be high.

We used the following materials and techniques in our method to ensure accuracy.

Standard materials. SRM 911a, which is certified to be 99.8 ±0.1% pure cholesterol, was used as the *primary reference material.* This function in a definitive method should be fulfilled by a high-purity, widely available, certified reference material, because the limit of accuracy that can be achieved with a definitive method is fixed by the accuracy with which the purity of the primary reference material is known. With SRM 911a as the primary standard material, the accuracy limit is the difference between the "true value" and the certified purity. However, no contribution to the overall bias of the definitive method is ascribed to this source of systematic error, because all cholesterol methods that are referenced to SRM 911a are equally subject to this error.

As the *internal standard,* the method involves the use of a preparation of cholesterol-d_7 containing less than 0.1% of cholesterol-d_0, an isotopic purity that is much greater than necessary. We detected only one chemical impurity, a C_{24} steroid. It was present to the extent of 1–2%, but its exact proportion was not determined because, in the absence of a specific interference from the impurity, the accuracy of the method is independent of the purity of the internal standard. The chemical impurity was not a potential source of interference in the measurements, because of its lower relative molecular mass. We found no evidence that as much as 0.1% of any other impurity might be present in the crystalline cholesterol-d_7.

Sampling technique. The CV for the aliquot-weighing technique for ethanol solutions, tested by measuring the radioactivity per gram of aliquot transferred, was <0.16%. Further evidence of the precision of this technique was obtained in the ID/MS within-day precision study, where this sampling technique was used for both the serum and the ethanol solution of cholesterol-d_7, and the CV of the complete results ranged between 0.16 and 0.24% (Table 3).

Wet-chemical techniques. Our method involves hydrolysis conditions that are somewhat more severe (i.e., the mixture is more alkaline and the reaction time is longer) than those usually used (*18*), in order to ensure complete ester hydrolysis and mixing of all the cholesterol-d_0 with the added cholesterol-d_7. Under these conditions, hydrolyzed mixtures consisted of a single phase, which assured the mixing of the two forms.

Experiments with [4-^{14}C]cholesterol showed that a single hexane extraction of the hydrolysis mixture sufficed, because about 99% of the cholesterol was extracted. We assumed that the ratio of cholesterol-d_7 and -d_0 in the extract would correspond to that in the hydrolyzed mixture.

The mixtures of cholesterol-d_7 and -d_0 extracted from samples and those prepared by combining weighed aliquots of the two stock standard solutions were treated similarly with *N,O*-bis(trimethylsilyl)acetamide for conversion into the cholesterol trimethylsilyl ethers. The reaction products were stored in the excess reagent, and aliquots of these mixtures underwent the ratio measurement. The weight ratios of many of these stored mixtures, redetermined by ion-intensity measurement and bracketing during many months, were always found to be reproducible.

Calibration of isotope ratio measurements. The previously published ID/MS methods for total cholesterol involved calibration procedures that are commonly used with ID/MS.

Bjorkhem et al. (*4*) plotted the measured peak heights (i.e., the relative ion-intensities) against the known weight ratios of the mixtures they prepared from their labeled cholesterol and their unlabeled cholesterol. The plot was used as a standard curve for converting the measured peak height ratio for a sample into a weight ratio. From the latter and the known weight of added labeled cholesterol, the cholesterol in the sample could be calculated.

On the other hand, Sieckmann et al. (*6*) measured the relative peak-heights of the masses of interest in their reference samples of labeled cholesterol and non-labeled cholesterol separately, calculated the amount of labeled cholesterol added from the relative peak heights measured on a standard solution of labeled cholesterol mixed with a solution containing a known amount of non-labeled cholesterol, and used these values—together with the relative peak heights they measured for each sample—to calculate the total cholesterol in each sample.

Gambert et al. (*7*) measured relative peak areas rather than peak heights, but otherwise followed measurement practices similar to those of Sieckmann et al.

Thus, by using these procedures, these workers relied on their mass spectrometers to provide ion-intensity ratios that were linear over a wide range of weight ratios, and throughout the time required for measuring all of the samples and standards. They also expected these measurements to be independent of the quantity of sample injected into the GC/MS.

In our ID/MS method the conditions were chosen to optimize precision. Measurements are made according to a strict protocol, used with samples prepared under restrictive conditions: (*a*) The ratios of labeled to unlabeled cholesterol were limited to a narrow range near 1:1, thus ensuring the attainment of linearity and providing optimum conditions for measurement of the ratios. Table 1 lists the weight ratios of the calibration mixtures used. Samples were mixed with cholesterol-d_7 in such proportions that the ratios to be measured were within the range of the calibration mixtures, which requires that the approximate total cholesterol content for each sample be obtained (by some routine method) before the ID/MS analysis is undertaken. (*b*) The two aliquots of each serum pool taken for analysis as part of a set were not identical in quantity, nor were the amounts of labeled cholesterol combined with them identical. Consequently, the intensity ratios observed for the two mixtures usually differ, as do the pairs of calibration mixtures needed for bracketing them (see Table 2). This procedure ensures that the effect of any error in a single calibration mixture is minimized. (*c*) Relatively large and uniform quantities of the mixtures of labeled and unlabeled cholesterol were injected into the GC/MS for ratio measurements, to minimize the possible effects of background at the masses of interest and to circumvent possible nonlinear response of the mass spectrometer ion source to sample size. (*d*) Sample measurements were made according to the bracketing protocol described. Adherence to the rule for valid measurements helps to ensure that the measurements are made while the instrumentation is in proper working order. Adherence to the rule for repeating the measurements of the samples and the same two calibration mixtures on a second day with the bracketing in reverse order minimizes the potential for systematic error such as that expected from gas-chromatographic column or instrument-memory effects. A preliminary estimate of the precision of the method was obtained from the analyses performed on Pools I–III (see Table 3).

Accuracy of the ID/MS method. The statistical analysis of the ID/MS results on Pools A–E, summarized in Table 5, shows the replication error in those measurements to be

characterized by a CV of 0.17% and the set-to-set component of variability by a CV of 0.32%; thus the variability of a single measurement from set-to-set is represented by a CV of 0.36%. Each serum average has a standard error (considering all causes of variability combined) of 0.16% CV.

The plots given in Figure 1 reveal that, within the variability described above, there are systematic differences in results between sets as well as between pools. These small systematic effects, which are variable in magnitude and direction, are more clearly evident in sets 3 and 4. They can be attributed to errors in the concentrations of the cholesterol-d_7 stock standard solutions prepared for each set. The source of this error might be in sampling a possibly non-homogeneous crystalline cholesterol-d_7, or in weighing it, or both. As a result there would be an error of similar magnitude in the quantity of cholesterol-d_7 added to each serum aliquot in a set, which would lead to errors of similar magnitude in the amount of total cholesterol found in each aliquot. The appearance of the concentration-dependent enlargement of the error illustrated in Figure 1 results from expressing the total cholesterol in terms of concentration. Because the precision of these results is already ample for a definitive cholesterol method, we did not investigate the source of this error.

The similarity of the values shown in Table 6, which we obtained by ID/MS analysis of the same samples, but using several different pairs of ions from the cholesterol-d_7 and -d_0 trimethylsilyl ethers, demonstrates the essential absence of interference with the intensity ratios measured at m/z 465 and 458; otherwise, at least some of these ratios would have deviated markedly. Even after noting that the second-day results obtained from the chemical ionization measurements are somewhat low, these additional results show that our methodology affords the high degree of specificity that is usually claimed from use of ID/MS.

A principal source of systematic error that may remain undetected in our results would be associated with the concentration of the stock standard solutions of cholesterol-d_0 used for preparing the calibration mixtures. The possible slight nonhomogeneity of the cholesterol-d_7 previously discussed and any error in weighing either the cholesterol-d_7 or -d_0 would contribute to that error. We believe the undetected systematic error (bias) in the results reported here does not exceed 0.5%.

This study was carried out as part of a program to develop definitive and reference methods, which was supported by the Food and Drug Administration under FDA contract number 74-50(0) and by the National Bureau of Standards.

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